CARDIOVASCULAR MEDICINE

Evidence for association of a common variant of the endothelial nitric oxide synthase gene (Glu^{298}→Asp polymorphism) to the presence, extent, and severity of coronary artery disease

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Background: Genetic variants of endothelial nitric oxide synthase (eNOS) could influence individual susceptibility to coronary artery disease.
Objective: To assess whether Glu^{298}→Asp polymorphism of the eNOS gene is associated with the occurrence and severity of angiographically defined coronary artery disease in the Italian population.
Methods: Polymerase chain reaction/restriction fragment length polymorphism analysis was done to detect the Glu^{298}→Asp variant of the eNOS gene in 201 patients with coronary artery disease and 114 controls. The severity of coronary artery disease was expressed by the number of affected vessels and by the Duke scoring system.
Results: The frequencies of the eNOS Glu/Glu, Glu/Asp, and Asp/Asp genotypes in the coronary artery disease group were significantly different from those of controls (45.3%, 38.8%, and 15.9% *v* 42.1%, 51.8%, and 6.1%, respectively; $\chi^2 = 8.589$, p = 0.0136). In comparison with subjects who had a Glu^{298} allele in the eNOS gene, the risk of coronary artery disease was increased among Asp/Asp carriers (odds ratio 2.9, 95% confidence interval 1.2 to 6.8, p = 0.01) and was independent of the other common risk factors (p = 0.04). There was a significant association between the eNOS Glu^{298}→Asp variant and both the number of stenosed vessels (mean (SEM), 2.3 (0.1) for Asp/Asp *v* 1.9 (0.1) and 1.8 (0.1) for Glu/Glu and Glu/Asp, respectively; p = 0.01) and the Duke score (56.1 (3.1) for Asp/Asp *v* 46.7 (2.0) and 46.1 (1.9) for Glu/Glu and Glu/Asp, respectively; p = 0.02).
Conclusions: Glu^{298}→Asp polymorphism of the eNOS gene appears to be associated with the presence, extent, and severity of angiographically assessed coronary artery disease.

Epidemiological studies indicate that hyperlipidaemia, hypertension, cigarette smoking, diabetes, and obesity are risk factors for coronary artery disease.[1–3] Control of these environmental risk factors has, however, been ineffective in completely predicting development of the atherosclerotic process, suggesting that specific genetic predisposition should be taken into account as well.[4 5]

Vascular endothelium modulates blood vessel wall homeostasis through the production of factors regulating vessel tone, coagulation state, cell growth, cell death, and leucocyte trafficking.[6] One of the most important endothelial cell products is nitric oxide (NO), which is synthesised from L-arginine by the enzyme endothelial nitric oxide synthase (eNOS).[7] NO plays a key role in the relaxation of vascular smooth muscle, inhibits platelet and leucocyte adhesion to the endothelium, reduces vascular smooth muscle cell migration and proliferation, and limits the oxidation of atherogenic low density lipoproteins.[8] Moreover, it has been shown that eNOS inhibition accelerates atherosclerosis in animal models, and that abnormalities of the endothelial NO pathway are present in humans with atherosclerosis.[9 10] This evidence suggests that NO may inhibit several key steps in the atherosclerotic process and that an alteration of NO production within the vascular endothelium could contribute to the pathogenesis of atherosclerosis. Thus eNOS could be a candidate gene for atherosclerosis.

Several polymorphisms have been identified in the eNOS gene, among which is one located in exon 7 (G984T) which modifies its coding sequence (Glu^{298}→Asp). Associations between this variant and coronary spasm, coronary artery disease, and acute myocardial infarction have been reported, but data on its relation with disease severity are lacking.[11 12] In this paper, we describe the associations between the Glu^{298}→Asp polymorphism of the eNOS gene and the occurrence and severity of angiographically defined coronary artery disease in the Italian population.

METHODS

Study population

We studied 201 patients consecutively admitted to our institution with angiographically proven coronary artery disease (more than 50% stenosis affecting at least one vessel) and 114 control subjects recruited from patients admitted for valve replacement, in whom angiographic examination excluded the presence of coronary artery disease. All the patients and controls were interviewed and data on smoking habits, hypertension, diabetes, dyslipidaemia, and family history of coronary artery disease were recorded. Informed consent was obtained from all patients and controls, as required by our ethics committee.

For coronary risk factors, the following definitions were used: subjects were defined as hypertensive if their blood pressure was > 140/90 mm Hg or if they were receiving any antihypertensive treatment; those with a history of diabetes or who were receiving any antidiabetic drugs were considered to be diabetic; those with a total plasma cholesterol concentration of ⩾ 5.70 mmol/l or a triglyceride concentration of ⩾ 2.26 mmol/l, or who were receiving lipid lowering drugs, were considered dyslipidaemic. Smoking history was coded as

Table 1 Demographic and clinical characteristics of coronary artery disease cases and controls

	CAD cases (n=201)	Controls (n=114)	p Value
Age (years) (mean (SEM))	59.8 (0.7)	56.7 (1.2)	0.02
Male sex	179 (89.0)	56 (49.1)	<0.0001
Hypertension	123 (61.2)	32 (28.1)	<0.0001
Diabetes	44 (21.9)	5 (4.4)	<0.0001
Dyslipidaemia	138 (68.7)	23 (20.2)	<0.0001
Smoking			<0.0001
Non-smokers	77 (38.3)	75 (65.8)	
Ex-smokers	91 (45.3)	25 (21.9)	
Current smokers	33 (16.4)	14 (12.3)	
Family history of CAD	89 (44.3)	28 (24.6)	0.0005
Number of diseased vessels			
One vessel	73	–	
Two vessels	65	–	
Three vessels	63	–	

Values are n (%) unless stated.
CAD, coronary artery disease.

Table 2 Genotype frequencies of Glu^{298}→Asp polymorphism in angiographically defined coronary artery disease cases and controls

	Glu/Glu	Glu/Asp	Asp/Asp	Total
CAD cases	91 (45.3)	78 (38.8)	32 (15.9)	201
Controls	48 (42.1)	59 (51.8)	7 (6.1)	114

Values are n (%).
χ^2=8.589, p=0.0136 for genotype.
CAD, coronary artery disease.

never, ex (for least six months), and current. A positive family history was the presence of a first degree relative with coronary artery disease at the age of ⩽ 55 years for men and ⩽ 65 years for women.

Angiographic study

All patients and controls underwent coronary angiography. Coronary stenosis was considered significant in the presence of a luminal diameter narrowing of ⩾ 50% of at least one epicardial coronary artery. The severity of coronary artery disease was expressed by the number of affected vessels (one, two, or three vessel disease) and also by means of the Duke scoring system[13]—a prognostic index that includes the number of diseased major vessels, the presence of left main coronary artery disease, the percentage narrowing of the major vessels, and involvement of the left anterior descending coronary artery, particularly when the proximal segment shows severe stenosis (⩾ 95%). The Duke score ranges from 0–100 (0 = no disease, 100 = the most severe disease).

Analysis of Glu^{298}→ Asp polymorphism on exon 7 of eNOS gene

Genomic DNA was extracted from samples of whole blood by standard methods.[14] The coding sequence variant was a G→T substitution at position 894 in exon 7 which determines the Glu to Asp amino acid substitution (in codon 298) in the mature eNOS protein. According to previously described procedure, genotyping of all subjects was performed by polymerase chain reaction amplification of exon 7 with the primers 5′-CATGAGGCTCAGCCCCAGAAC-3′ (sense) and 5′-AGTCAATCCCTTTGGTGCTCAC-3′ (antisense) followed by *Mbo*I restriction enzyme digestion for 16 hours at 37°C.[12] In the presence of a T at nucleotide 894 which corresponds to Asp 298, the 206 base pair (bp) polymerase chain reaction product is cleaved into two fragments of 119 and 87 bp. The products of the digestion process were highlighted by electrophoresis on a 1.5% agarose gel.

Table 3 Odds ratio for coronary artery disease among individuals heterozygous or homozygous for the Asp^{298} variant

Genotype	Reference group	Odds ratio (95% CI)	p Value
Asp/Asp	Glu/Glu	2.4 (1 to 5.9)	0.03
Asp/Asp	Glu/Glu + Glu/Asp	2.9 (1.2 to 6.8)	0.01

CI, confidence interval.

Table 4 Relative risks of coronart artery disease by coronary risk factors and by Glu^{298}→Asp polymorphism of the eNOS gene

Risk factor	Relative risk (95% CI)	p Value
Age	1 (0.9 to 1.0)	0.09
Male sex	7.6 (3.4 to 16.6)	<0.0001
Smoking	1.4 (0.5 to 3.9)	0.48
Hypertension	2.9 (1.4 to 5.8)	<0.01
Diabetes	10.6 (3.1 to 36.5)	<0.0001
Dyslipidaemia	8.3 (4.1 to 16.6)	<0.0001
Family history of CAD	2.2 (1.1 to 4.6)	0.03
Asp/Asp *v* Glu/Glu and Glu/Asp genotypes	2.9 (1 to 8.8)	0.04

CAD, coronary artery disease; CI, confidence interval.

Statistical analysis

All statistical analyses were conducted with the Statview statistical package, version 5.0.1 (SAS Institute). Data are expressed as mean (SEM). Differences between the means of the two continuous variables were evaluated by Student's *t* test. Differences in non-continuous variables, genotype distribution, and the Hardy–Weinberg equilibrium were tested by χ^2 analysis. One way analysis of variance was used to analyse the relations between genotypes and the general characteristics and severity of coronary artery disease, in terms of the number of diseased vessels and the Duke score. Logistic regression analysis was used to assess the independent effect of each risk factor on the occurrence of coronary artery disease. A probability value of $p < 0.05$ was considered to be significant.

RESULTS

Comparison of the two study groups

Demographic and clinical characteristics of patients and controls were given in table 1. The prevalence of atherogenic risk factors (including age, sex, hypertension, diabetes, cigarette smoking, dyslipidaemia, and a family history of coronary artery disease) was significantly higher in the patient group.

Distribution of the Glu^{298}→Asp polymorphism of the eNOS gene

Although the distribution of genotypes in both coronary artery disease cases and controls satisfied the Hardy–Weinberg equilibrium, the Glu^{298}→Asp polymorphism in exon 7 of the eNOS gene was significantly associated with the presence of coronary artery disease in our patients (table 2). The proportion of Asp^{298} homozygotes was 15.9% in the coronary artery disease cases and 6.1% in control subjects (χ^2 = 8.589, p = 0.0136).

In comparison with Glu^{298} homozygotes, the odds ratio (OR) for coronary artery disease associated with the Asp/Asp genotype was 2.4 (table 3). Because Glu/Asp carriers were not at

Table 5 General and clinical characteristics of patients in each Glu^{298}→Asp genotype

Variable	Glu^{298}→Asp genotype			p Value
	Glu/Glu (n=91)	Glu/Asp (n=78)	Asp/Asp (n=32)	
Age (years) (mean (SEM))	60.0 (1.0)	59.9 (1.1)	58.5 (1.7)	0.74
Sex (% male)	90.1	85.9	93.7	0.44
Non-smokers (%)	34.4	37.7	34.4	0.87
Ex-smokers (%)	50	42.8	50	
Current smokers (%)	15.6	19.5	15.6	
Hypertension (%)	63.7	62.3	51.6	0.47
Diabetes (%)	22	24.7	16.1	0.62
Dyslipidaemia (%)	67	72.4	64.5	0.65
Family history of CAD (%)	46.1	44.2	40.6	0.86
Number of vessels involved (mean (SEM))	1.9 (0.1)	1.8 (0.1)	2.3 (0.1)	0.01

CAD, coronary artery disease.

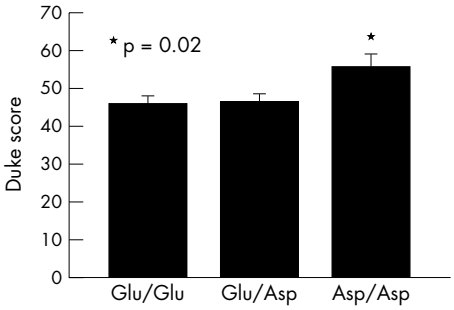

Figure 1 Glu^{298}→Asp polymorphism of the eNOS gene and the severity of coronary artery disease assessed by the Duke scoring system.

increased risk of coronary artery disease (OR 0.66, 95% confidence interval 0.4 to 1.1), the odds ratios associated with the Asp/Asp genotype were therefore computed with Glu/Glu + Glu/Asp considered as the reference group. As shown in table 3, in comparison with individuals homozygous and heterozygous for Glu^{298}, the odds ratio for coronary artery disease among Asp/Asp carries was 2.9. Multivariate analysis showed that the Asp/Asp genotype was an independent risk factor for coronary artery disease (table 4).

Glu^{298}→Asp polymorphism of the eNOS gene and severity of coronary artery disease

The relations between several variables and Glu^{298}→Asp genotype were studied in coronary artery disease patients (table 5). We did not find any association between the genotype and hypertension, dyslipidaemia, diabetes, smoking status, or family history of coronary artery disease.

We found a significant association between the Glu^{298}→Asp variant of the eNOS gene and the severity of the disease in terms of the number of stenosed vessels. eNOS Glu^{298}→Asp polymorphism was also associated with the extent and the severity of coronary artery disease evaluated by the Duke scoring system (mean (SEM): 56.1 (3.1) for Asp/Asp, 46.7 (2.0) for Glu/Asp, and 46.1 (1.9) for Glu/Glu; p = 0.02) (fig 1).

DISCUSSION

We report the association between the common Glu^{298}→Asp polymorphism of the eNOS gene and the occurrence of coronary artery disease in the Italian population. We found an excess of homozygosity for the Asp^{298} variant among coronary artery disease cases compared with controls, and the risk of developing coronary artery disease was about threefold higher for Asp^{298} homozygotes than in persons with a Glu^{298} allele in the eNOS gene. Multivariate analysis showed that this association was independent of other factors related to coronary artery disease risk. Our study provides the first evidence for an association between Glu^{298}→Asp polymorphism and the extent and severity of coronary artery disease.

There was the expected clustering of coronary artery disease risk factors among cases. However, we did not detect an association between the Glu^{298}→Asp polymorphism and any of these possibly confounding variables.

Glu^{298}→Asp polymorphism of the eNOS gene and risk of atherosclerosis related disease

Up to now, Glu^{298}→Asp polymorphism of the eNOS gene has been linked to an increased risk of stroke, coronary atherosclerosis, and acute myocardial infarction.[15 16] Previous studies from Japan and the UK have already suggested a role for Glu^{298}→Asp polymorphism in the development of coronary atherosclerosis, with the excess risk being confined to Asp^{298} homozygosity,[12 17] as in our study. These studies, however, also showed that the genotype frequency of Glu^{298}→Asp polymorphism can vary substantially among different populations. For example, while in the Japanese population this polymorphism could only explain a small part of the genetic susceptibility to acute myocardial infarction, as the Asp/Asp genotype was present in only five of 226 patients (2.2%),[17] in the UK the Asp/Asp genotype was found in 107 of 298 patients (35%) and in 45 of 249 patients (18.1%) with coronary artery disease and acute myocardial infarction, respectively.[12] Our genotype frequencies in both cases and controls were in agreement with those recently reported by Lembo and colleagues among Italian subjects who had atherosclerotic plaques on their carotid arteries and control subjects without carotid plaques.[15] In that study, Asp^{298} homozygosity was an independent risk factor for the development of carotid plaques, but no association was found between Glu^{298}→Asp polymorphism of the eNOS gene and the degree of involvement of the various segments of the carotid arteries. Nevertheless, it is important to emphasise that some groups have failed to find any relation between the Asp^{298} variant and the risk of atherosclerosis.[18–21] Elbaz and colleagues even found a significant association between the Glu/Glu genotype and the risk of brain infarction.[21]

Functional significance of the Glu^{298}→Asp polymorphism of the eNOS gene

The fact that in our study the risk for coronary artery disease was confined to Asp^{298} homozygotes suggested that homozygosity for aspartic acid in position 298 could produce a reduction in the amount or enzymatic activity of eNOS. If the Asp^{298} variant of eNOS leads to altered NO synthesis, this could provide a mechanism for both its increased prevalence among patients with coronary artery disease and its association with the extent and severity of the disease. Several experimental studies have in fact shown that a reduction in the endothelial

production of NO appears to be critical for the evolution, progression, and clinical manifestations of the atherosclerotic process.[22 23] It is noteworthy that in our study an association was observed between the Glu[298]→Asp polymorphism and both the number of diseased vessels and the Duke scoring system—a prognostic index that also includes the percentage narrowing of the major vessels and the anatomical localisation of the stenosis. Thus our data suggested that the Asp[298] variant of eNOS could contribute to the generalised architecture of the vessels. This hypothesis is supported by in vivo evidence that eNOS mutant mice display a paradoxical increase in wall thickness accompanied by a hyperplastic response of the arterial wall after carotid artery ligation.[24] This suggests that a primary defect in the NOS/NO pathway may promote abnormal remodelling and pathological changes in vessel wall morphology associated with atherosclerosis. Thus it is possible that in the process of atherosclerotic remodelling of adult human vessels, alterations in NO production resulting from the substitution of Glu[298] with Asp[298] could have a major impact on smooth muscle cell migration and proliferation. Indeed, despite the apparently conservative nature of the Glu→Asp amino acid substitution, there is evidence that a similar substitution in other enzymes can alter protein function.[25] Recently, Philip and colleagues showed that enhanced vascular responsiveness to phenylephrine was associated with the Asp[298] allele,[26] and a significant reduction in endothelium dependent dilatation has been correlated with Glu[298]→Asp polymorphism in early pregnancy.[27] On the other hand, a study by Schneider and associates appeared to exclude an effect of eNOS Glu[298]→Asp polymorphism on endothelium dependent vasodilatation.[28] Furthermore, Sofowora and colleagues reported that Asp[298] homozygotes excreted significantly less nitrate/nitrite than Glu[298] homozygotes without affecting nitric oxide mediated vascular responses.[29] Thus the impact of Glu[298]→Asp polymorphism on endothelial NO function remains to be clarified.

Finally, genetic contributions of eNOS to plasma NO metabolite concentrations have been recently reported. The mutant allele of the T[786]→C polymorphism in the promoter region of the eNOS gene has been associated with a reduced promoter activity and endothelial synthesis of NO, both of which predispose to coronary spasm in the Japanese population.[30] Moreover, Yoshimura and colleagues found that the T[786]→C variant is in linkage disequilibrium with the eNOS gene intron 4b/a polymorphism,[31] which is also reported to be involved in smoking dependent coronary artery disease,[32] suggesting that the T[786]→C mutation underlies the functional characteristics of the intron 4a allele. It is not known whether the associations that we reported in this study reflect a hypofunctional enzyme or linkage disequilibrium between the Glu[298]→Asp polymorphism and another functional variant within the eNOS gene or another gene.

Conclusions

We observed that the Glu[298]→Asp polymorphism of the eNOS gene is associated with the presence, extent, and severity of angiographically assessed coronary artery disease in the Italian population. Because this polymorphism has also recently been associated with carotid atheroma in the same population, more studies are needed to investigate whether the Glu[298]→Asp polymorphism of the eNOS could represent an useful genetic marker to identify individuals prone to the development of atherosclerotic diseases.

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